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Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation

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Background. Epithelial-mesenchymal transformation (EMT) plays an important role in embryonic development and tumorigenesis and has been described in organ remodeling during fibrogenesis. In the kidney, EMT can be induced efficiently in cultured proximal tubular epithelium by coincubation of transforming growth factor (TGF)-β1 and epidermal growth factor (EGF). Recently, we also have observed overexpression of basic fibroblast growth factor-2 (FGF-2) protein and mRNA in human kidneys with marked interstitial fibrosis. The aims of the present study were to compare the effects of FGF-2 as a facilitator of EMT in tubular epithelial cells with EGF and TGF-β1. We analyzed the morphogenic effects of the three cytokines on four different aspects of EMT: cell motility, expression and regulation of cellular markers, synthesis and secretion of extracellular matrix (ECM) proteins as well as matrix degradation.

Methods. Cell motility was studied by a migration assay and cell differentiation markers were analyzed by immunofluorescence and immunoblots. In addition, regulation of the epithelial adhesion molecule E-cadherin and fibroblast-specific protein 1 (FSP1) were analyzed by luciferase reporter constructs and stable transfections. ELISAs for collagen types I and IV and fibronectin were used for ECM synthesis, and zymograms were utilized for analysis of matrix degradation.

Results. FGF-2 induced cell motility across a tubular basement membrane in two tubular cell lines. All three cytokines induced the expression of vimentin and FSP1, but only FGF-2 and TGF-β1 reduced cytokeratin expression by immunofluorescence. These effects were most demonstrable in the distal tubular epithelial cell line and were confirmed by immunoblot analyses. Expression of E-cadherin was reduced by 61.5 ± 3.3% and expression of cytokeratin by 91 ± 0.5% by TGF-β1 plus FGF-2. Conversely, the mesenchymal markers α-smooth muscle actin (SMA) and FSP1 were induced with FGF-2 by 2.2 ± 0.1-fold and 6.8 ± 0.9-fold, respectively. Interestingly, de novo expression of the mesenchymal marker OB-cadherin was induced only by FGF-2 and EGF but not by TGF-β1. All three cytokines stimulated FSP1 and decreased E-cadherin promoter activity. FGF-2 also induced intracellular fibronectin synthesis but not secretion, the latter of which was stimulated exclusively by TGF-β1. Finally, zymographic analyses demonstrated that FGF-2 induced MMP-2 activity by 2.6 ± 0.5-fold and MMP-9 activity by 2.4 ± 0.1-fold, providing a mechanism for basement membrane disintegration and migratory access of transforming epithelium to the interstitium.

Conclusions. FGF-2 makes an important contribution to the mechanisms of EMT by stimulating microenvironmental proteases essential for disaggregation of organ-based epithelial units. Furthermore, the expression of epithelial and mesenchymal marker proteins seems to be affected at the promoter level.

Interstitial fibrosis and tubular atrophy are structural markers of end-stage renal failure. The degree of tubulointerstitial scarring is an excellent prognostic marker for progressive organ failure [1]. Fibroblasts are thought to be the main effector cells in renal fibrogenesis. These cells reside within the cortical interstitial spaces of the kidney or in perivascular areas. When fibroblasts activate, some phenotypically convert to myofibroblasts that are marked by the de novo expression of α-smooth muscle actin (SMA) [2]. Not all collagen-producing fibroblasts make this conversion, and it has been speculated that some of the scattered SMA^+ cells in the interstitium may be vascular smooth muscle cells [3]. $FSP1^+$ cells proliferate and synthesize extracellular matrix products such as fibronectin and collagen types I, III, IV and V. However, the origin of these cells is still unclear. Whereas the current paradigm sees fibroblasts as descendants of resident interstitial fibroblasts or perivascular adventitial cells [4],

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they may be replenished, in part, from bone marrow stromal cells [5] or tubular epithelial cells [6].

Tubular epithelial cells have several important functions in renal fibrogenesis. Initially, they react to proteinuria by synthesizing chemoattractants such as endothelin-1 (ET-1), RANTES (regulated upon activation, normal T cell expressed and secreted), and monocyte chemoattractant protein-1 (MCP-1) [7]. All three moieties are strong attractants for monocytes/macrophages that help to form early tubulointerstitial infiltrates. These interstitial infiltrates are thought to release profibrogenic morphokines such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), which in turn activate and cause fibroblasts to divide. At another level, tubular epithelial cells may stimulate the activation of fibroblasts directly by their secretion of TGF-β1 and PDGF. Finally, tubular epithelium may contribute to the formation of extracellular matrix. Creely and coworkers demonstrated that TGF-β induced collagen type I production in rat tubular epithelial cells [8]. Similarly, TGF-β1 stimulated fibronectin and proteoglycan synthesis in rabbit proximal tubule cells [9]. Collagen type I synthesis in tubular epithelial cells also is stimulated by hyperglycemia [10]. We have cloned a murine fibroblast-specific protein FSP1 [11]; FSP1 is a 11.5-kD cytoskeletal protein belonging to the calmodulin-S100-troponin C superfamily of intracellular calcium binding proteins that are involved in cell motility and mesenchymal phenotype [12]. In mouse models of antiglomerular and antitubular basement membrane disease, selected tubular cells express FSP1 de novo. We interpret this finding as a sign of potential epithelial-mesenchymal transformation (EMT).

The process of EMT is characterized by a loss of apical-basal polarity, cellular adhesion molecules, and cell-cell junctions of involved epithelium [13]. Epithelium in transition develop front and back-end polarity and become mobile. The intermediate filament profile changes from cytokeratin to vimentin, the F-actin fibers reform to a mesenchymal format, and some begin to become SMA^+. Moreover, the cells reverse the types of extracellular matrix they synthesize from basement membrane type to interstitial matrix components [14]. Okada et al were able to demonstrate that FSP1 expression can be induced most effectively by a combination of EGF and TGF-β1 [2]. Furthermore, this group demonstrated an early role of FSP1 in EMT since antisense oligonucleotides for FSP1 inhibited EMT at an early stage. Ng and coworkers demonstrated that rat tubular epithelial cells acquire SMA expression in a model of interstitial fibrosis and fulfill the criteria of a mesenchymal cell by electron microscopy [6]. The change of cell phenotype was accompanied by disruption of the basal membrane. Similarly, Nadasdy and coworkers analyzed human end-stage kidneys and found single cells or small cell clusters within the tubulointerstitial space that expressed mesenchymal and epithelial markers simultaneously [15]. Recently, Jinde et al described tubular phenotypic changes indicating EMT in human glomerulonephritis [16].

We have recently described the potential profibrogenic role of FGF-2 in human renal fibrogenesis [17, 18]. The aim of the present study was to analyze the effects of FGF-2 on EMT in tubular epithelial cells in vitro and compare these effects with the effects induced by EGF and TGF-β1. Four different aspects of EMT were analyzed: cell motility, expression and regulation of epithelial and mesenchymal markers, synthesis of ECM proteins, and degradation of basal membrane.

METHODS

Materials

Human recombinant EGF, FGF-2 and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA), as were the neutralizing antibodies to EGF (goat polyclonal), FGF-2 (goat polyclonal), and TGF-β (chicken polyclonal). Antibodies to EGF (rabbit polyclonal) and FGF-2 (mouse monoclonal) and to receptors for EGF (EGFR) (rabbit polyclonal) and FGF-2 (FGFR-1) (rabbit polyclonal) were from Calbiochem-Novabiochem (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The following antibodies were used for cell characterization, immunocytochemistry and immunoblot studies: anti-cytokeratin (mouse monoclonal which reacts with keratins 5, 6, 8, and 17; Dako, Carpinteria, CA, USA), anti-E-cadherin (rat monoclonal; Takara, Shiga, Japan), anti-ZO-1 (rat polyclonal; Chemicon, Temecula, CA, USA), anti-vimentin (mouse monoclonal; Boehringer Mannheim, Mannheim, Germany), anti-SMA (mouse monoclonal; Paesel + Lorei, Wiesbaden, Germany), and OB-cadherin (goat polyclonal; Santa Cruz). Synthesis and characterization of the anti-FSP1 antibody have been described before [11]. For the enzyme-linked immunosorbent assay (ELISA) technique, antibodies to collagen type I (rabbit polyclonal; Biodesign, Kennebunk, ME, USA), collagen type IV (rabbit polyclonal; Biodesign) and fibronectin (rabbit polyclonal; Sigma) were used. Trypsin-EDTA (0.05/0.02%, wt/vol), Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium and fetal calf serum (FCS) were obtained from Gibco BRL Ltd. (Paisley, Scotland, UK). Cell culture dishes were from Becton Dickinson (Franklin Lakes, NJ, USA).

Cell culture

The tubular epithelial cell line NP-1 was generated from freshly isolated tubuli from a C57BL/6J mouse. Cortical slices were dissected and dissociated by treatment with collagenase. Tubule fragments were washed and resuspended in defined medium (DMEM:Ham's F12;

1:1 vol/vol) containing insulin (0.5 μg/mL), triiodothyronin (1 μmol/L), transferrin (5 μg/mL), EGF (10 ng/mL), dexamethasone (50 nmol/L) and 5% FCS. Cell characterization was done by immunocytochemistry. Cells were seeded in microchamber slides and incubated for 12 hours. They were subsequently fixed with ethanol/acetic acid (50:50, vol/vol) for 20 minutes at 4°C, washed and incubated with the primary antibody for two hours at room temperature. Cells were then washed again with phosphate-buffered saline (PBS) and incubated for 60 minutes at room temperature with the appropriate FITC-conjugated secondary antibody. Control cultures were incubated without application of the primary antibody. Cell characterization was performed using the following antibodies: anti-cytokeratin, anti-E-cadherin, anti-ZO-1, anti-vimentin, anti-SMA, and anti-FSP1. All cells were positive for cytokeratin, ZO-1, and E-cadherin, and were negative for vimentin, FSP1 and SMA. In addition, segment-specific lectin binding using *Dolichus biflorus* agglutinin (DBA) as a marker for distal and collecting tubules revealed positivity in all cells, whereas it was negative for the proximal tubule marker *Lotus tetraglonolobus* (LTA). Furthermore, NP-1 cells did not stain positively for alkaline phosphatase (data not shown). These data indicate that NP-1 cells represent a cell type of the distal tubule.

Mouse proximal epithelial tubule (MCT) cells, NIH/3T3 fibroblasts and the mouse kidney fibroblast line TFB, which have been characterized previously [19, 20], were cultured in DMEM supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 U/mL).

Immunoblot analyses for EGFR and FGFR-1

Immunoblots for expression of EGFR and FGFR-1 were performed as described before [17]. Briefly, lysates from MCT and NP-1 tubular epithelial cells were obtained by lysis with a detergent-based buffer containing 0.4% sodium deoxycholate, 1% NP-40, 1.9% EGTA and 10 mmol/L Tris (pH 7.4). 100 μg of total cellular protein were run on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane (Hybond™ ECL™; Amersham, Braunschweig, Germany) by electroblotting. Membranes were stained with Ponceau red to control for adequate transfer and equal loading. After blocking, the membrane was incubated with the respective antibody [anti-EGF and anti-FGF-2 at a concentration of 1:100, anti-EGFR at 1:80, and anti-FGFR-1 (flg) in a concentration of 1:40] followed by the secondary antibody [anti-goat (Santa Cruz) or anti-rabbit (Amersham) horseradish-peroxidase-linked, at concentrations of 1:12,000 and 1:3000, respectively]. Positive reaction products were identified by chemiluminescence (ECL; Amersham) according to the manufacturer's protocol.

Assessment of cell motility

Cell motility was assessed by migration assay. In order to mimic the microenvironment of tubular basement membrane and renal interstitium, polyvinyl-pyrolidone-free (PVPF) polycarbonate membranes with 8 μm pores (Neuro Probes, Inc., Gaithersburg, MD, USA) were coated with type IV collagen on the upper side (50 μg/mL) and with type I collagen on the lower side (50 μg/mL). The lower wells of a 48-well Boyden chamber were filled with DMEM medium alone. Wells were covered with the coated membrane sheet and 2×10^4 cells/well were added into the upper chamber either in DMEM alone or with FGF-2 in concentrations of 0.1, 1.0, and 10 ng/mL. EGF and TGF-β1 (5 ng/mL each) was used as a positive control. The Boyden chamber was incubated for four hours at 37°C to allow possible migration of cells through the membrane into the lower chamber. Membranes were stained with Hema3® stain according to manufacturer's recommendations (Biochemical Sciences, Inc., Swedesboro, NJ, USA). Cells that migrated through the membrane were counted using a counting grid, which was fitted into the eyepiece of the phase contrast microscope. All experiments were repeated three times.

Expression of epithelial and mesenchymal markers

The expression of epithelial and mesenchymal markers was analyzed by indirect immunofluorescent labeling and immunoblot analyses. For indirect immunofluorescence labeling, NP1 and MCT tubular epithelial cells were plated at a density of 5×10^6/mL on 10-well multitest slides (ICN, Costa Mesa, CA, USA) and serum-deprived for 24 hours. Subsequently, cytokines were added at a concentration of 1 ng/mL. After 72 hours immunocytochemistry was performed as described above using indirect immunofluorescence to stain for the epithelial markers cytokeratin and for the mesenchymal markers vimentin and FSP1. Anti-cytokeratin antibody was used at 1:20, anti-vimentin at 1:10, and anti-SMA at 1:50. The secondary FITC-labeled antibodies (anti-mouse or anti-rat; both from Dako) were used at 1:50. In a second series of experiments, cell density was decreased to 1×10^6/mL and cells were incubated with 1 ng/mL FGF-2 for 72 hours. Additional staining with the Hoechst dye H33258 to identify nuclei was performed to facilitate cell counting in that experiment. For each group, 500 cells were determined microscopically using an Axiophot S100 microscope (Zeiss, Jena, Germany) by a blinded investigator and the percentage of positive cells was calculated. Each experiment was repeated three times independently. Again, neutralizing antibodies to the respective cytokines were added in the concentrations as above to control for specificity. In addition, the effect of an irrelevant IgG (anti-HLA-DR) was examined in a concentration of 10 μg/mL.

Further quantification of expression of epithelial and mesenchymal markers was obtained by immunoblot analyses for the epithelial markers E-cadherin and cytokeratin and the mesenchymal markers SMA, FSP1 and OB-cadherin in NP1-cells. Cells were cultured in flasks until reaching confluence and were then stimulated with the appropriate cytokine or cytokine combination in a total concentration of 1 ng/mL. Furthermore, in order to study dose dependency, FGF-2 was added to confluent cell cultures at concentrations of 0.1, 1.0 and 10.0 ng/mL for 72 hours. Total cellular protein was harvested after 24, 48, 72, or 96 hours of incubation using the detergent-based buffer described above. One hundred nanograms of protein were run on 18 or 15% SDS-PAGE gel and blotted overnight. Primary antibodies were used in the following concentrations: anti-E-cadherin 1:100, anti-cytokeratin 1:100, anti-SMA 1:100, anti-FSP1 1:15,000, and anti-OB-cadherin 1:200. The secondary horseradish peroxidase-linked antibodies were used at the following concentrations: anti-goat 1:12,000, anti-rabbit 1:3000, anti-mouse 1:1500, and anti-rat 1:1000 (the latter two from Amersham). Positive reaction products were again identified by chemiluminescence using a Fluor-S™ Multi-imager (Bio-Rad, Hercules, CA, USA) and densitometric analysis was performed using Multi-Analyst™ software (Bio-Rad). Values of densitometric analyses were corrected for protein loading. All experiments were repeated three times with independent stimulations.

Stable transfections with FSP1 and E-cadherin luciferase minigenes

For further analyses of FSP1 and E-cadherin regulation, stable transfections in NP1 cells were performed. The characterization of the FSP1 promoter has been described in detail before [21]. The luciferase reporter construct pBK1800luc containing an 1800 bp fragment of the 5′ flanking region of the *FSP1* gene was transfected into NP1 cells using $CaPO_4$ as described elsewhere [11]. Cotransfections with pMCNeobGal (Stratagene) in a molar ratio of 10:1 favoring pBK1800luc were used for selection. In pBK1800luc, the luciferase gene is placed under the control of the FSP1 promoter. This construct demonstrated activity exclusively in fibroblasts [11]. Twenty-four hours after transfection, cells were subjected to selective medium (DMEM + 400 μg/mL Genetecin™) for 14 days and then subcloned by limiting dilution. For analysis of E-cadherin promoter activity, 3500 bp of the E-cadherin promoter (generous gift of Dr. J. Behrens, Essen, Germany) was cloned into the pXP-2 luciferase vector placing the luciferase gene under control of the E-cadherin promoter (pUvo3500luc) [22]. Since the activity of pUVO3500luc was not entirely specific for epithelial cells as already described [22], a series of luciferase reporter minigenes were engineered by restriction enzymes creating fragments of 495 (pUvo495 luc), 214 (pUvo214luc), 107 (pUvo107luc) and 17 (pUvo17luc) bp upstream of the transcriptional start site. All constructs were confirmed by sequence analysis. These deletion fragments were transiently transfected into MCT, NP1 and NIH/3T3 cells using co-transfections with pCH110 (Pharmacia, LKB Biotechnology, Piscataway, NJ, USA), a vector expressing β-galactosidase, for normalization as described [11]. pSV_2luc containing the SV40-promoter/enhancer served as a positive control. Since pUvo214luc displayed by far the strongest activity in both tubular epithelial cell lines (49.1 ± 4.3% of pSV_2luc in NP1 and 42.4 ± 3.4% in MCT compared to 9.1 ± 1.4% in NIH/3T3 fibroblasts), this construct was stably transfected into NP1 cells as described above. Stable transfectants were stimulated by FGF-2, EGF, and TGF-β1, or combinations of TGF-β1 and EGF or FGF-2 at a total concentration of 1 ng/mL for 48 hours before measurement. All luciferase activities were measured by cell lysis in KPO_4-DTT with 1% Triton X-100 and assays of the supernatants in a luminometer (Mikrolumat CB 96P; Berthold, Bad Wildbad, Germany). Light units of stable transfectants were normalized for cell number. Stimulations were repeated five times independently and results are given as relative percentage compared to unstimulated stable transfectants.

Analysis of extracellular matrix synthesis

Extracellular matrix (ECM) synthesis was evaluated by ELISAs for collagen types I and IV and for fibronectin in both cell lines. In addition, immunoblot analyses for intracellular fibronectin synthesis were performed in NP1-cells. ELISAs of supernatants were performed as described previously [17]. Briefly, 8×10^3 cells were plated per well and cells were made quiescent by incubation in serum-free medium for 24 hours. Next, cytokines were added in a concentration of 1 ng/mL followed by incubation for 72 hours. Both 50 μg/mL ascorbic acid and 50 μg/mL propionitrile (both from Sigma) were added in experiments evaluating collagen synthesis. Supernatants were transferred to a Maxisorp™ plate (Nunc) and incubated overnight at room temperature. Plates were subsequently dried for two hours and blocked with 3% milk. Incubation with 50 μL of the primary antibody (anti-collagen type I at a concentration of 1:200, anti-collagen type IV at 1:10,000, and anti-fibronectin at 1:5000) was followed by washing twice with PBS/0.1% Tween and incubation with the secondary antibody (anti-mouse-IgG-AP and anti-rabbit-IgG-AP (both Boehringer Mannheim), both used at a concentration of 1:1000). After two additional wash steps 100 μL chemiluminescence-ELISA substrate (Boehringer Mannheim) was added and quantitation was performed in a luminometer (Mikrolumat CB 96P; Berthold) using MikroWin™ software (Mikrotek, Overath, Germany). Nonspecific binding was determined by incubation with the secondary antibody

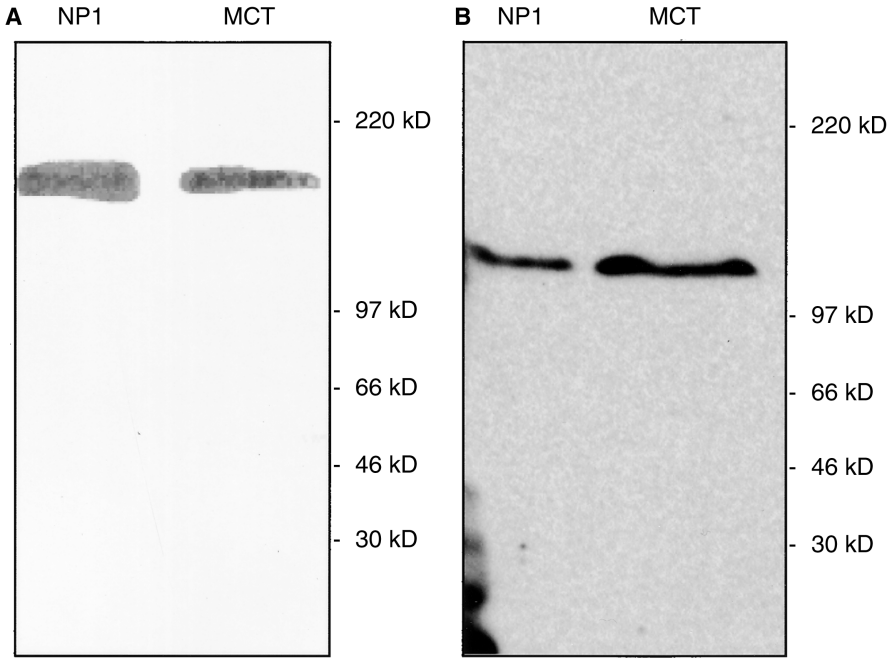

Fig. 1. Immunoblot analyses for epidermal growth factor (EGF) receptor (*A*, 170 kD) and basic fibroblast growth factor (FGF) receptor 1 (*B*, 120 kD) in MCT (mouse proximal tubular epithelial cell line) and NP1 cells (distal tubular epithelial cell line). Both cell lines express the two receptors.

only. The value was normally less than 5% of the total chemiluminescence and was subtracted from each assay. All assays were performed in triplicates and repeated five times. Standardization was obtained for the collagen type I ELISA using rat tail type I collagen (Sigma). Limit of detection was 1 ng/mL, working range 10 to 1000 ng/mL. Mouse collagen type IV isolated from basement membrane of Engelbreth-Holmes-Swarm mouse sarcoma (Sigma) served as standardization for the type IV collagen. Human fibronectin (Sigma) was used as standard for the fibronectin ELISA; the working range of this ELISA was 0.1 to 10 μg/mL with a limit of detection of 10 ng/mL. All standards were purchased from Sigma. Values were corrected for cell counts that were performed after the transfer of supernatants and were expressed in ng or $\mu g/10^3$ cells.

Immunoblot analyses of intracellular fibronectin were performed as described above. Cells were stimulated with the appropriate cytokine or cytokine combination at a concentration of 1 ng/mL for 24, 48, 72 and 96 hours. Furthermore, in order to study dose dependency, FGF-2 was added to confluent cell cultures at concentrations of 0.1, 1.0 and 10.0 ng/mL for 72 hours. The anti-fibronectin antibody was used in a concentration of 1:5000, the secondary antibody at 1:3000. Human fibronectin served as positive control. Positive reaction products were identified by chemiluminescence using a Fluor-S™ Multiimager (Bio-Rad) and densitometric analysis was performed using Multi-Analyst™ software (Bio-Rad). Values of densitometric analyses were corrected for protein loading.

Matrix degradation

Effects of the three cytokines on gelatinase [matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9)] were analyzed by zymography in both tubular epithelial cell lines. Cells were plated at a density of 8×10^4/mL and growth-arrested in Iscoves medium for 24 hours. Cells were subsequently stimulated with the appropriate cytokine at a concentration of 1 ng/mL for 72 hours. Cell supernatants were ×10 concentrated and equal amounts of total protein were loaded on SDS gel containing 1.0 mg gelatin per mL, and electrophoresis on non-reducing SDS-10% polyacrylamide gels (Biorad) was performed. After 30 minutes of incubation in renaturation buffer containing 2.5% Triton X-100, gelatinolytic activity was detected by an 18-hour incubation step in 5 mmol/L $CaCl_2$, 50 mmol/L Tris/HCl, pH 7.5 at 25°C before Coomassie Brilliant Blue staining of the gels (0.5% Coomassie blue in 40% methanol/10% acetic acid for 1 hour). Destaining was performed in the same buffer devoid of Coomassie blue for 15 minutes. The presence of the metalloproteinases was indicated by an unstained proteolytic zone of the substrate. Molecular sizes of the bands displaying enzymatic activity were identified by comparison to standard proteins and to purified MMPs (both from Calbiochem). Quantification was achieved by densitometric analysis as described above.

Statistical analysis

All values are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine statistical differences between growth factor treated

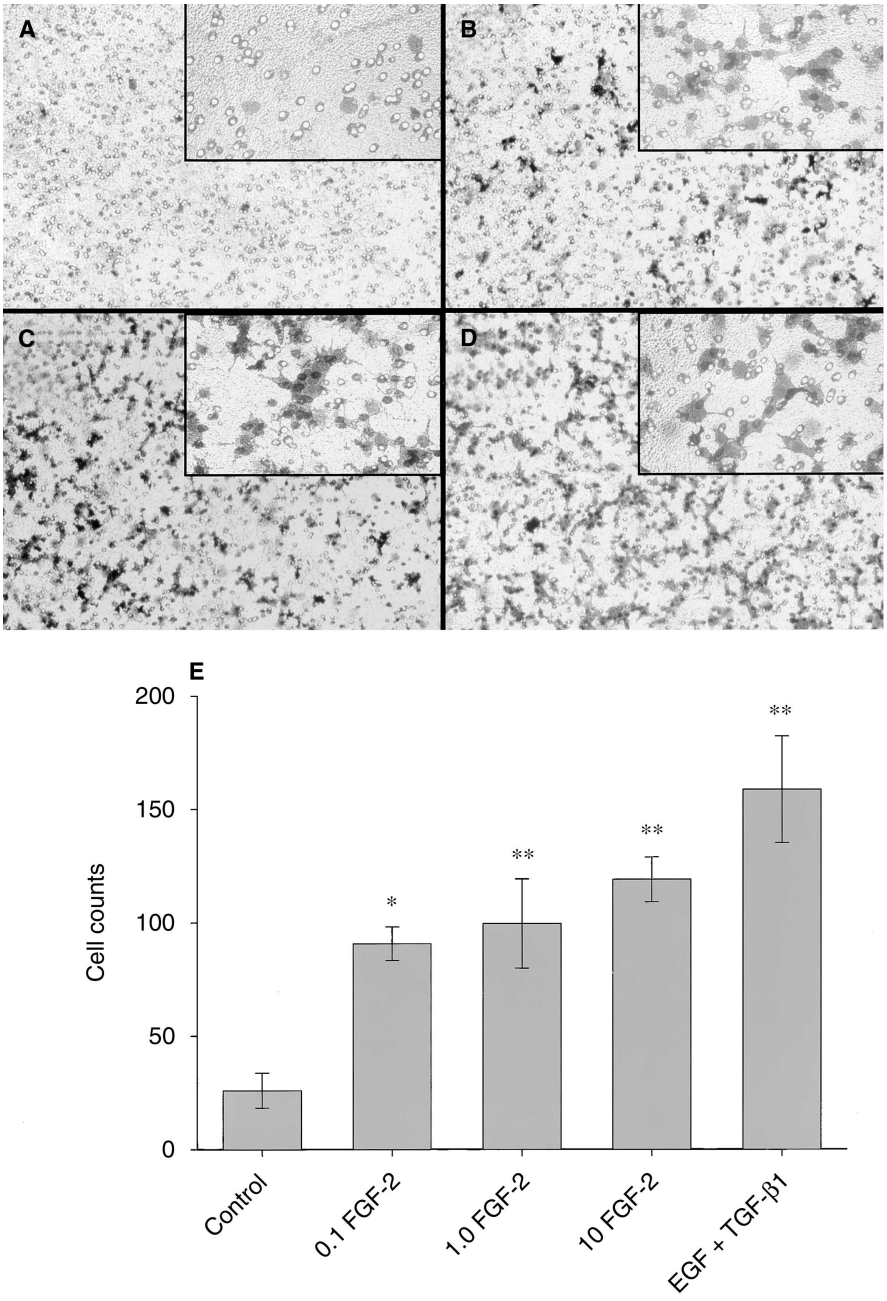

Fig. 2. Assessment of cell migration in NP1 tubular epithelial cells. Whereas in controls relatively few cells were able to migrate through the artificial basement membrane (*A*), stimulation with FGF-2 resulted in a robust upregulation of migrated cells (*B* depicts stimulation with 1.0 ng/mL and *C* 10 ng/mL). The most robust stimulation was obtained with the combination of EGF and TGF-β1 (used in this experiment at the concentrations of 5 ng/mL for each cytokine). Magnification ×100 and ×400 (inlets). $*P < 0.05$; $**P < 0.01$.

groups and controls using Sigma-Stat™-software 2.03 (Jandel Scientific, San Rafael, CA, USA). Bonferroni's method was applied to control for multiple testing. *P* values <0.05 were considered significant.

RESULTS

Immunoblot analyses for EGFR and FGFR-1

Immunoblot analyses demonstrated that the tubular epithelial cell lines MCT and NP1 expressed type 1 receptors for FGF-2 and the EGF receptor making these cells susceptible to the biologic effects of the respective cytokine (Fig. 1). In addition, FGF-2 protein was detectable in MCT and NP1-cells by immunoblot analysis (not shown), but not EGF protein. Thus, both cell lines expressed the receptors for FGF-2 and EGF, though only FGF-2 was synthesized by these cells.

Induction of cell motility

Basic fibroblast growth factor-2 induced cell motility as assessed by the migration assay in a dose dependent fashion. Figure 2 illustrates the results obtained with NP-1 cells. In addition to the promotion of migratory capacity, cells developed a more fibroblastoid morphol-

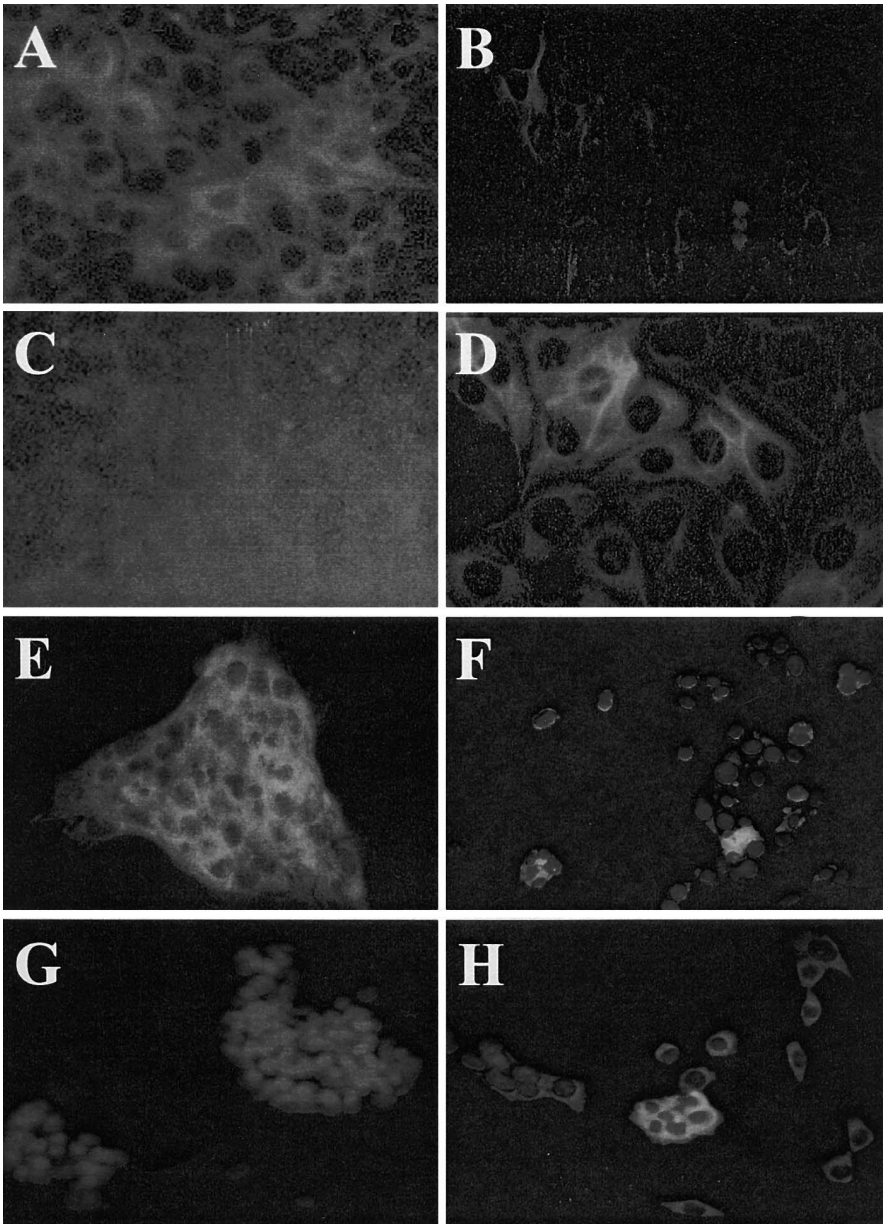

Fig. 3. Indirect immunofluorescence stainings for the epithelial marker cytokeratin (*A*, *B*, *E* and *F*), and the mesenchymal marker vimentin (*C*, *D*, *G* and *H*) in NP1 tubular epithelial cells. Cells were stimulated for 72 hours with EGF, FGF-2 or TGF-β1 at a concentration of 1 ng/mL. Only results obtained with FGF-2 are depicted. Panels A–D depict the results obtained at high cell density, panels E–H at low cell density. Stimulation with FGF-2 (1 ng/mL) resulted in a down-regulation of cytokeratin and an up-regulation of vimentin protein expression. These changes were more prominent at lower cell density. Magnifications ×600 (A–D) and ×400 (E–H).

ogy (see inlets in Fig. 2 B–D vs. A of control). In MCT cells, FGF-2 induced cell migration even more robustly and dose dependently (163.5 ± 16.2 cells at 0.1 ng/mL FGF-2, 181.7 ± 20.6 at 1 ng/mL FGF-2, and 252 ± 23.7 at 10 ng/mL FGF-2 vs. 39.5 ± 10.3 of controls and 325.2 ± 21.3 for EGF + TGF, P for all <0.01).

Differential effects on phenotypic cell markers

Expression of phenotypic cell markers was first studied by indirect immunofluorescence for the epithelial marker cytokeratin, as well as for the mesenchymal markers vimentin and FSP1 in NP1 cells. Cytokeratin expression was down-regulated after 72 hours by FGF-2 and TGF-β1 (Fig. 3), whereas EGF had no significant effect (not shown). The number of cytokeratin positive cells decreased from 98.7 ± 2.0% to 38.5 ± 3.5% (FGF-2) and 31.6 ± 5.1% (TGF-β1), respectively, but was unchanged after incubation with EGF alone (94.5 ± 3.1%). Conversely, all three cytokines resulted in increased expression of the two mesenchymal markers: Vimentin positivity increased to 74.5 ± 5.2% after induction by EGF, to 79.3 ± 4.3% after stimulation with FGF-2, and to 82.3 ± 6.1% after addition of TGF-β1 (controls were 25.4 ± 4.1% positive). Similarly, FSP1 expression was increased to 32.5 ± 3.2%, 35.1 ± 4.0, and 41.3 ± 3.9% after stimulation with EGF, FGF-2 and TGF-β1, respectively (controls were only 12.4 ± 3.5% positive). Addition of neutralizing antibodies to the respective cytokine

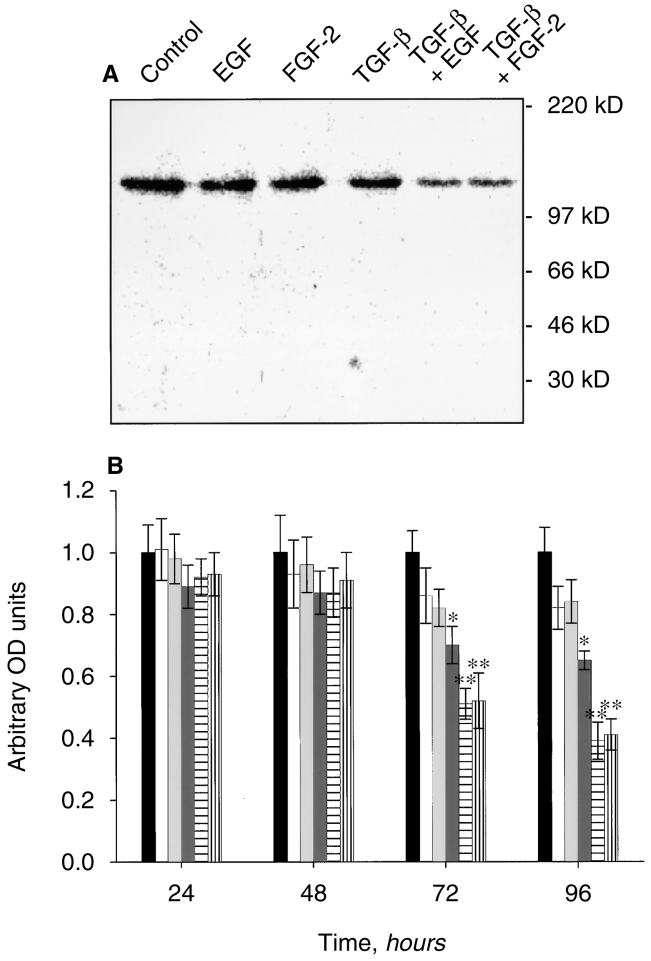

Fig. 4. Representative Western blot analyses and densitometric analyses for E-cadherin expression in NP1 cells. Part A depicts a typical blot obtained after stimulation for 96 h. Part B summarizes the results of three blots after three independent stimulations after 0, 24, 48, 72, and 96 hours. Symbols are: controls (■), and stimulations with EGF (□), FGF-2 (▨), TGF-β1 (▩), TGF-β1 + EGF (▤), or TGF-β1 + FGF-2 (▥). Whereas EGF and FGF-2 had only moderate effects on E-cadherin expression, TGF-β1 resulted in a significant reduction in expression after 72 and 96 hours. These effects were potentiated by addition of EGF or FGF-2. $^{*}P < 0.05$, $^{**}P < 0.01$.

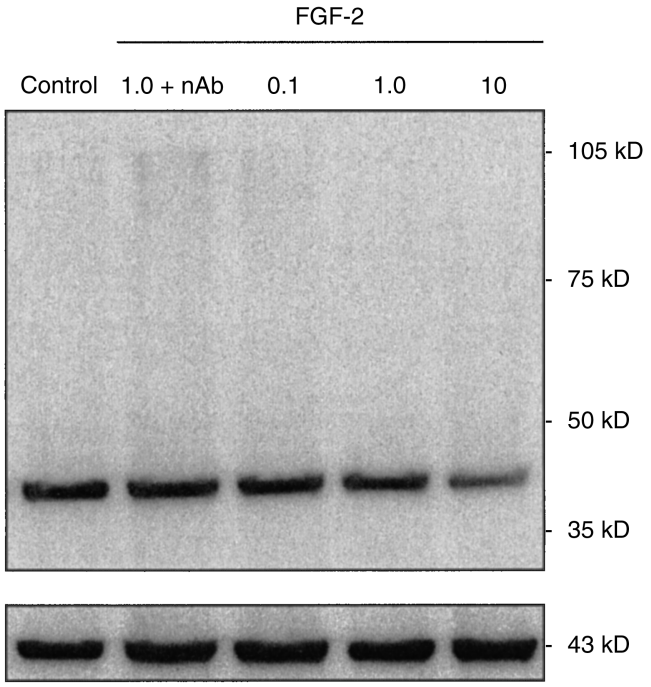

Fig. 5. FGF-2 results in dose dependent down-regulation of the epithelial cell marker cytokeratin by Western blot analysis. NP-1 cells were stimulated for 72 hours and cytokeratin expression was assessed by immunoblot. Addition of 10 ng/mL FGF-2 resulted in the most robust down-regulation whereas addition of a neutralizing antibody (nAb) prevented the effect. Actin (43 kD) was used in this experiment to control for equal protein loading (lower bands).

abolished its effects specifically, whereas the addition of a non-relevant IgG had no effect. Furthermore, the effects of FGF-2 on cytokeratin and vimentin expression were studied at lower density since the cell density may have an effect on the expression of epithelial or mesenchymal marker proteins. As shown in Figure 3 E–H, the cytokine reduced cytokeratin and increased vimentin expression even more compared to high density experiments (reduction of cytokine positive cells from 94.4 ± 2.9 to 26.3 ± 4.7 and increase of vimentin expressing cells from 5 ± 1.7 to 83.3 ± 4.6). Performing the same studies in MCT cells resulted in qualitatively similar changes albeit somewhat less pronounced (data not shown).

In order to further analyze these effects quantitatively, immunoblot analyses for the epithelial markers cytokeratin and E-cadherin, and for the mesenchymal markers FSP1, SMA and OB-cadherin were performed in NP1 cells (Figs. 4 to 8). Confirming the results of indirect immunofluorescence staining, cytokeratin expression was robustly decreased in a time-dependent fashion by TGF-β1 and FGF-2, whereas EGF did have no decreasing effect (not shown). FGF-2 had similar effects as TGF-β1 (reduction of cytokeratin expression by 62.1 ± 2.2 and 68.3 ± 5.2%, respectively). The dose dependency of FGF-2 on cytokeratin expression is shown in Figure 4. Regarding E-cadherin expression, EGF and FGF-2 reduced its expression (maximal reduction by 18.0 ± 7.2% after 96 h incubation with EGF) though only TGF-β1 decreased the expression significantly (maximal reduction by 35.3 ± 4.2%; Fig. 5). The effects of TGF-β1 were potentiated by either EGF or FGF-2 (decrease of up to 61.5 ± 3.3% after 96 h).

α-Smooth muscle actin is a cytoskeletal protein that is expressed in vascular smooth muscle cells and (activated) fibroblasts. Its expression was induced by FGF-2 and TGF-β1 (Fig. 6). FGF-2 resulted in a robust increase in SMA beginning at 24 hours and reaching a peak at 48 hours (increase by 17.4-fold). Conversely, induction by TGF-β1 was not significant until 48 hours after addi-

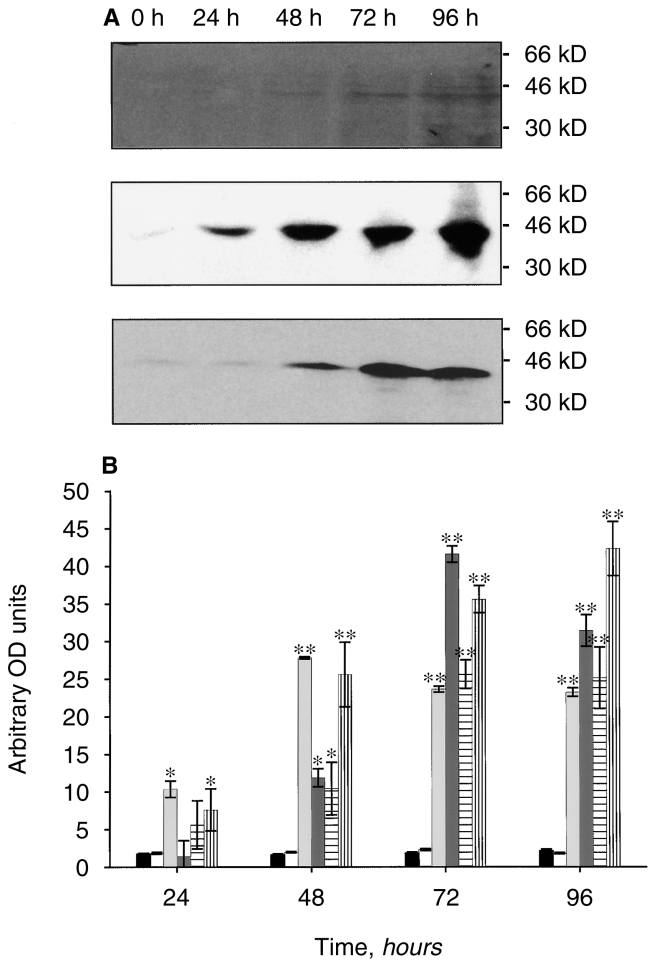

Fig. 6. Immunoblot analyses for α-smooth muscle actin (SMA) expression in NP1-cells. (*A*) Three typical blots for SMA (43 kD) after stimulation with EGF (upper panel), FGF-2 (middle panel) and TGF-β1 (lower panel). (B) Summary of the densitometric analyses of three independent stimulations with EGF (□), FGF-2 (▨), TGF-β1 (■), TGF-β1 + EGF (▤) and TGF-β1 + FGF-2 (▥). Controls are depicted in (■). EGF had no significant effect on α-smooth muscle actin expression, whereas FGF-2 and TGF-β1 increased its expression up to 40-fold. * $P < 0.05$, ** $P < 0.01$.

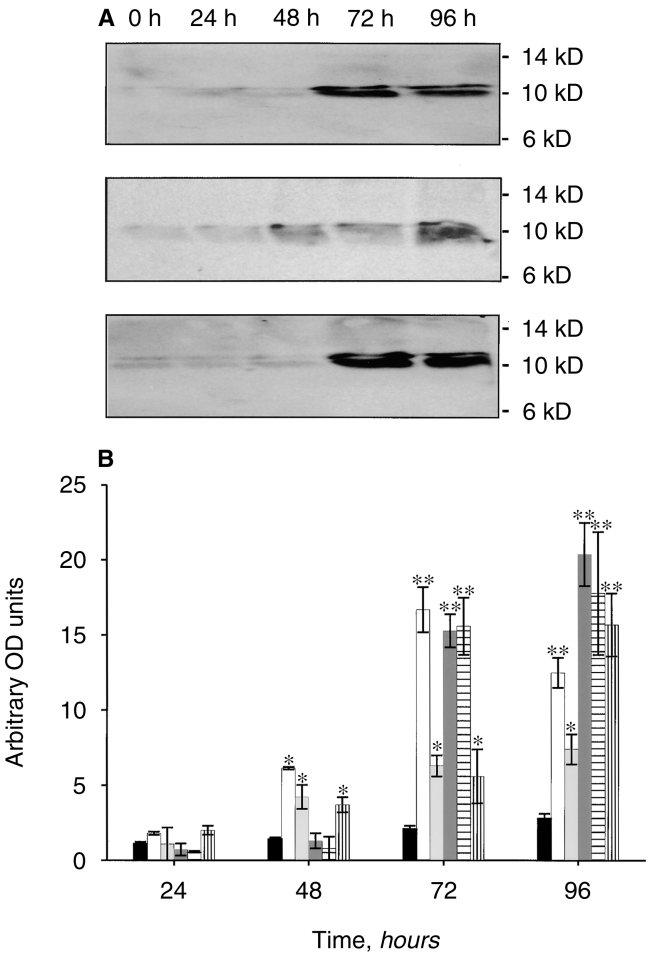

Fig. 7. Representative Western blots and summary of densitometric analyses for FSP1 in NP1-cells. Cells were stimulated for 0, 24, 48, 72, and 96 hours. (*A*) Typical blots after stimulation with EGF (upper panel), FGF-2 (middle panel) and TGF-β1 (lower panel). (*B*) Results of densitometric analyses of three independent experiments. Controls are shown in (■). EGF (□) and TGF-β1 (▨) had the most robust effects on FSP1 expression. Conversely, FGF-2 (▥) induced FSP1 less robustly. In addition, the last two columns display the effects of TGF-β1 + EGF (▤) and TGF-β1 + FGF-2 (▥). * $P < 0.05$, ** $P < 0.01$.

tion of the cytokine and peaked after 72 hours (increase by 23.1-fold). The combination of TGF-β1 and FGF-2 had similar effects as the single cytokines but had additive effects after 96 hours (increase by 20.2-fold). EGF by itself, on the other hand, did not induce synthesis of SMA, though in combination with TGF-β1 its effects were counterbalanced by the latter cytokine. However, regarding FSP1 expression, EGF exerted more robust effects than FGF-2 and resulted in an increase of up to 8.0-fold after 72 hours (Fig. 7). TGF-β1, again had the most pronounced effects after 96 hours (induction by 7.3-fold). The combination of TGF-β1 + EGF also increased FSP1 expression, but the combination of TGF-β1 with FGF-2 decreased the effects of TGF-β1. Finally, we analyzed the effects of all three cytokines on the expression of OB-cadherin (cadherin-11), a member of the family of non-classical cadherins that are expressed on cells with mesenchymal phenotype [23]. De novo synthesis of OB-cadherin was induced by EGF and FGF-2 in a time-dependent manner but not by TGF-β1 (Fig. 8).

Direct effects on promoter activity

For further analysis of cytokine-mediated effects on phenotypic markers, we next stably transfected luciferase reporter constructs containing active 5′-flanking regions of the FSP1 and E-cadherin genes. The results are summarized in Figure 9. E-cadherin promoter activity was inhibited maximally by the combination of TGF-β1 and FGF-2 (24.5 ± 2.1% of control). Conversely, FSP1 promoter was stimulated most robustly by EGF + TGF-β1 (216.8 + 5.9% compared to unstimulated control). Thus,

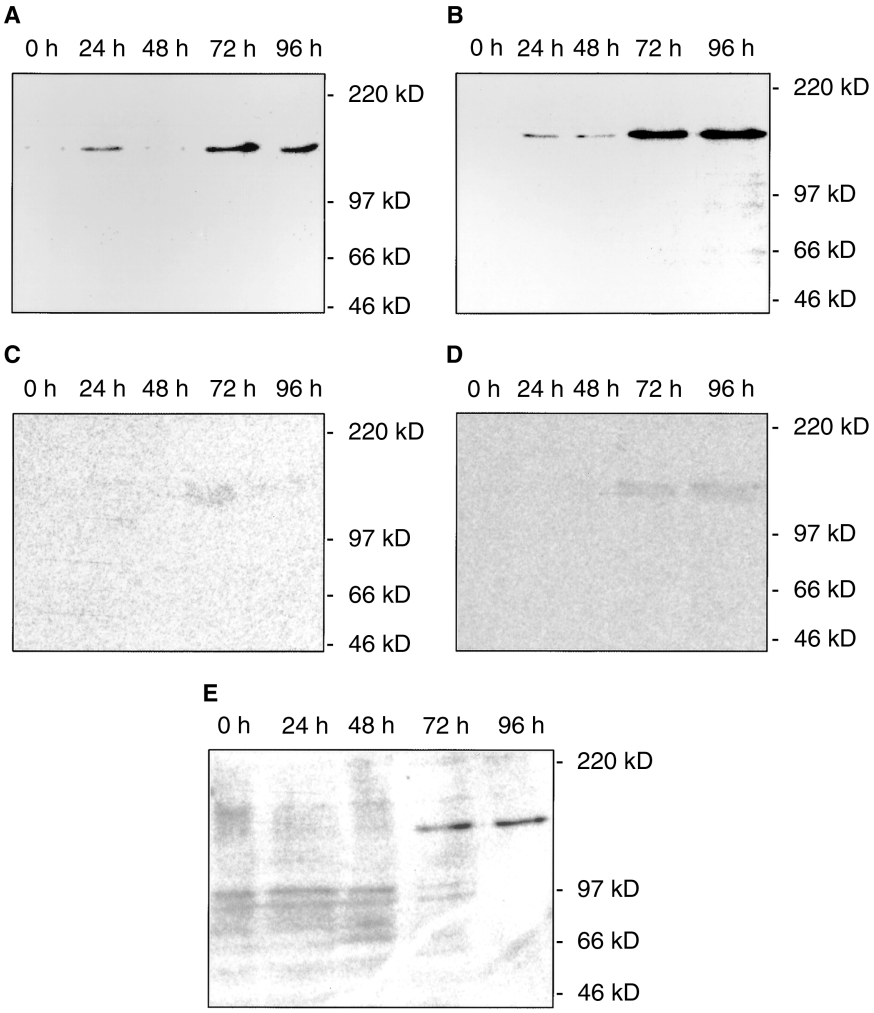

Fig. 8. Representative Western blot demonstrating the effects of EGF, FGF-2, and TGF-β1 on the expression of OB-cadherin in NP1 distal tubular epithelial cells. De novo protein synthesis for OB-cadherin is shown after incubation with EGF (*A*), FGF-2 (*B*), and TGF-β1 + FGF-2 (*E*) starting at 24 hours after incubation. Conversely, only minimal effects were observed after incubation with TGF-β1 (*C*) and the combination of TGF-β1 + EGF (*D*).

similarly to what was observed at the protein level, all three cytokines reduced E-cadherin promoter activity and promoted FSP1 expression. There is a certain discrepancy between the robust reduction of the E-cadherin promoter activity and the minor down-regulation of protein expression that could be explained by the long half life of the molecule on the cellular surface [24].

Effects of cytokines on extracellular matrix synthesis

The effects of the three cytokines and the combinations of TGF-β1 with EGF and FGF-2 on the secretion of collagen type I, collagen type IV and fibronectin are depicted in Figure 10. In MCT cells, collagen type IV secretion was stimulated up to 29.7 ± 3.3-fold and fibronectin release was increased up to 9.0 ± 0.6-fold by the combination of FGF-2 and TGF-β1. FGF-2 by itself had only marginal effects as did EGF. There were no significant effects of FGF-2 and EGF on secretion of type I and type IV collagens in both cell lines. Conversely, collagen type I secretion was induced up to 4.1 ± 0.5-fold and collagen type IV secretion was stimulated up to 5.9 ± 0.6-fold by TGF-β1 alone.

The effects on fibronectin synthesis in NP1 were in addition examined by immunoblot analyses. These blots demonstrated de novo expression of fibronectin protein induced by all three cytokines (Fig. 11). Thus, there is a discrepancy between the induction of intracellular fibronectin synthesis by EGF and FGF-2 and the lack of effect of these two cytokines on fibronectin secretion. In addition, the dose dependency of FGF-2 on fibronectin protein synthesis was demonstrated (Fig. 11F). Conversely, fibronectin synthesis and secretion were stimulated by TGF-β1 and the cytokine combinations that included TGF-β1, indicating that TGF-β1 is possibly required for secretion of this matrix protein.

Induction of matrix degradation

All three cytokines induced type IV collagen degrading enzymes MMP-2 and MMP-9 in both cell lines as determined by gel zymography. In MCT cells MMP-2 was induced up to 1.5 ± 0.2-fold compared to control (P not significant). MMP-9 was induced up to 1.6-fold ($P = 0.78$). The effects of the three cytokines were much

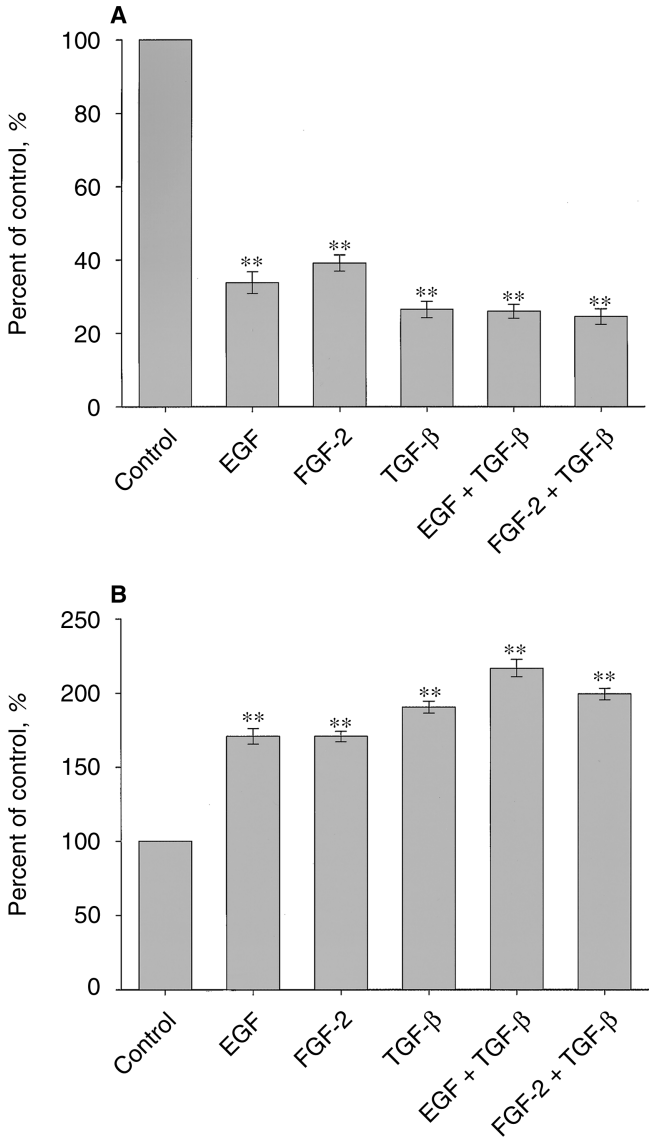

Fig. 9. Relative activity of the E-cadherin (*A*) and FSP-1 (*B*) promoter after stimulation with 1 ng/mL EGF, FGF-2, TGF-β1 alone or in combination. NP1 tubular epithelial cells were stably transfected and incubated with the relative cytokine for 48 h. Combinations of TGF-β1 + EGF (for FSP1) and TGF-β1 + FGF-2 (for E-cadherin) had the most robust effects on promoter activity. Values are given in percent of unstimulated controls.

more robust in NP1 cells (Fig. 12). MMP-2 was induced up to 5.53 ± 0.5-fold by FGF-2+TGF-β1, MMP-9 up to 5.5 ± 0.24-fold by EGF+TGF-β1 ($P < 0.001$ for both). TGF-β1 caused a 4.33 ± 0.51-fold increase in MMP-2 and a 3.1 ± 0.34-fold increase in MMP-9 ($P < 0.001$ for both). Conversely, EGF and FGF-2 significantly induced MMP-9 but not MMP-2 ($P = 0.08$ for EGF and $P = 0.12$ for FGF-2). Thus, again, FGF-2 had comparable effects to EGF, and potentiated TGF-β1–induced effects. However, it should be noted that activation of MMPs is tightly regulated for obvious reasons and that

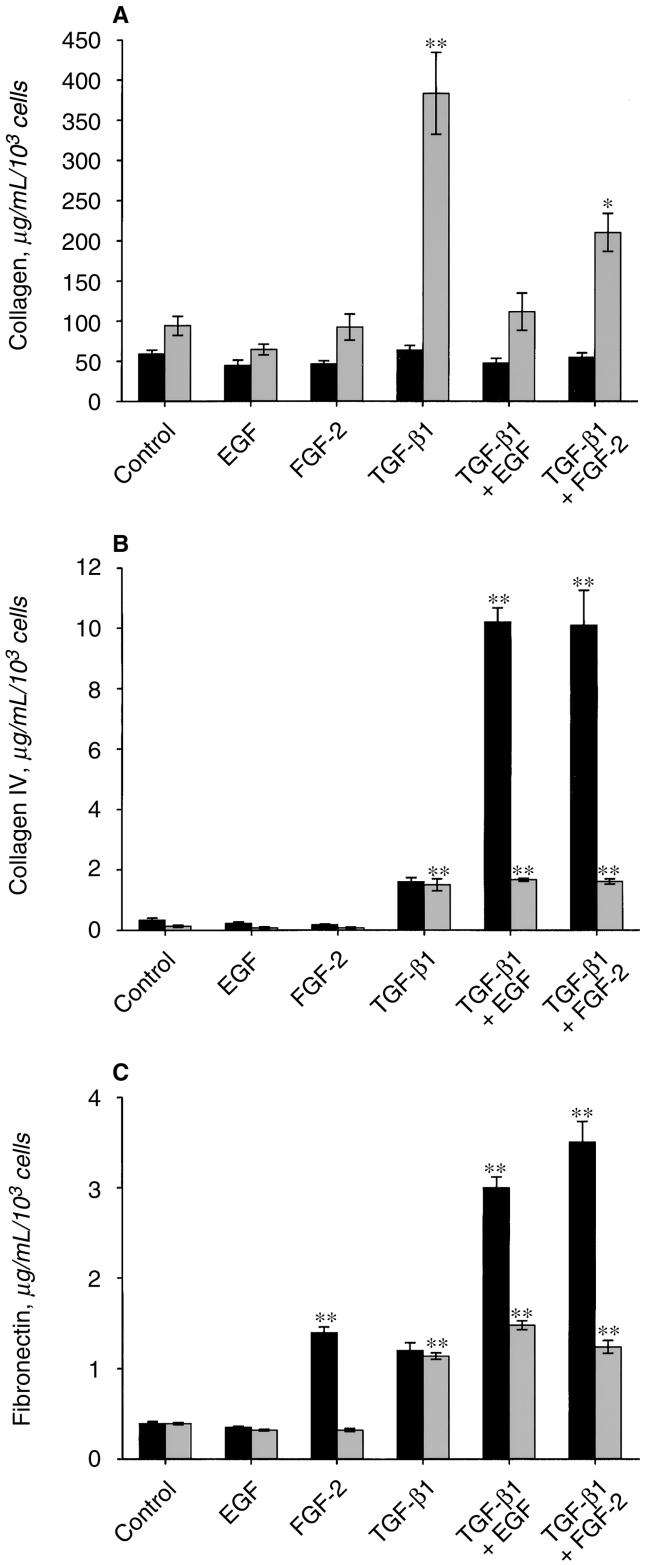

Fig. 10. ELISAs for collagen types I (*A*), IV (*B*) and fibronectin (*C*) in NP1 cells after stimulation for 72 hours. TGF-β1 had the most robust influence on collagen type I secretion. Coadministration of FGF-2 reduced this effect, and coadministration of EGF abolished it. Conversely, the effects of TGF-β1 on collagen type IV and on fibronectin secretion were potentiated by EGF and FGF-2 whereas the two cytokines had no noticeable effect by themselves. Symbols are: (■) MCT; (▒) NPI cells. *$P < 0.05$; **$P < 0.01$.

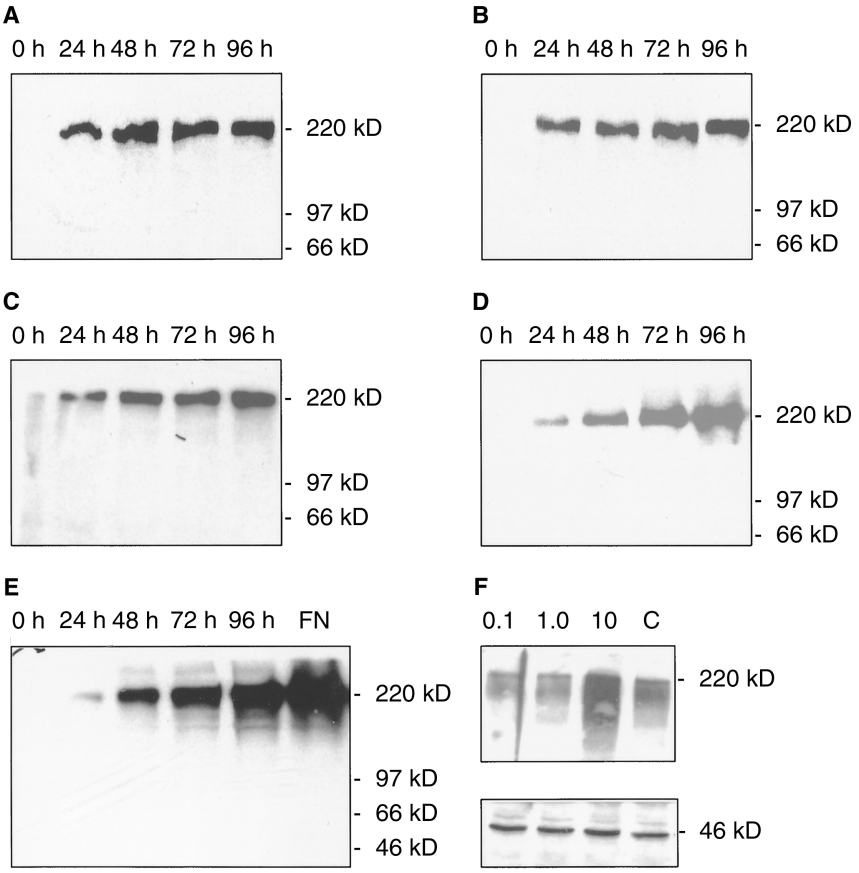

Fig. 11. Immunoblot analyses for fibronectin in NP1-tubular epithelial cells after stimulation with EGF (*A*), FGF-2 (*B*), TGF-β1 (*C*), TGF-β1 + EGF (*D*), and TGF-β1 + FGF-2 (*E*). Fibronectin standard (FN) is depicted in addition in E. Expression of fibronectin was increased by all three cytokines considerably starting 24 h after stimulation. The dose dependency of FGF-2 on fibronectin expression is depicted in part F (simulation for 72 h).

MMPs are secreted as latent form. We did not observe any additional bands that could indicate activated MMP-2 or MMP-9. Thus, activation is still required after secretion for these two enzymes to become active.

DISCUSSION

The differentiation state of a cell is not static but displays plasticity depending on its environment [13]. The extracellular matrix plays a particularly important role in modulating this plasticity. Definitive epithelia from organs such as the thyroid gland or lens epithelium convert into a mesenchymal phenotype when cultured in three-dimensional collagen gels [25]. Moreover, vascular smooth muscle cells alter from a contractile to a synthetic phenotype at contact with fibronectin or collagen type I [26]. In addition to extracellular matrix, cytokines also are integral facilitators of plasticity called cellular transdifferentiation. TGF-β is one of the most important cytokines in EMT [27]. Most forms of embryonic EMT rely on the presence of TGF-β, for example, transdifferentiation of epithelial cells of the mammary gland [28] and prostate [29]. The combination of TGF-β and EGF has been identified as an efficient cytokine combination in driving conversion of tubular epithelial to mesenchymal cells [2], whereas Fan and coworkers described tubular transdifferentiation induced by high doses of TGF-β1 [30]. Recently, Yang and Liu used a similar approach as we did to dissect the key events in TGF-β1 induced EMT [31]. Of particular significance is their finding that these in vitro findings can be recapitulated in vivo in the mouse model of unilateral ureteral obstruction.

Epithelial growth factor can induce phenotypic changes converting epithelium to a mesenchymal phenotype [32] and may promote cell motility in mammary epithelial cells [33]. Expression of EGF and EGFR is increased in a rat model of interstitial fibrosis and in human chronic renal diseases [34, 35]. Exciting studies by Terzi et al demonstrated a beneficial effect of EGF receptor mutations indicating a possible role of the EGF system in fibrogenesis [36], although this was could not be confirmed for all models [37]. FGF-2, on the other hand, has an important function for the development of mesenchymal cells [38]. It induces transdifferentiation of retinal epithelial cells into neuronal retinal cells [39] and has been implicated in regulating the cell phenotype of primary epithelium [40]. Increased expression of FGF-2 has been described in animal models of lung and liver fibrosis. Injections of FGF-2 into rats for up to 13 weeks resulted in focal segmental glomerulosclerosis and increase of the peritubu-

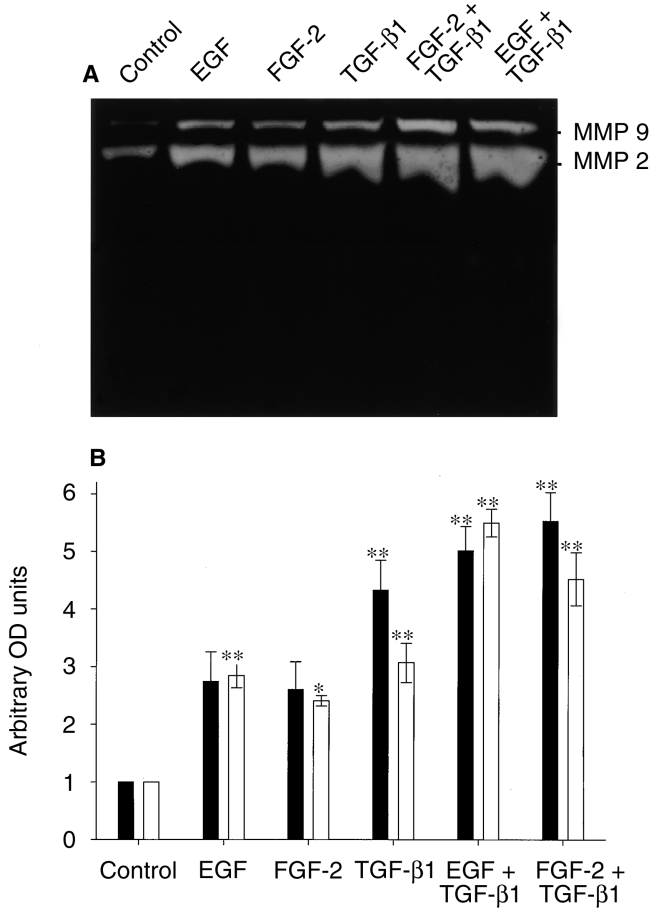

Fig. 12. Gelatin zymography of MCT and NP1 cells after stimulation with EGF, FGF-2, TGF-β1 or combinations including TGF-β1. MMP-2 and MMP-9 were visualized by negative staining with Coomassie brilliant blue dye solution. (*A*) A representative zymogram. (*B*) Proteolytic bands were scanned densitometrically and percentages of control values were calculated from 3 independent experiments. Symbols are: (■) MCT; (□) NPI; $*P < 0.05$; $**P < 0.01$.

lar interstitium [41]. Furthermore, interstitial overexpression of the cytokine has been described in a mouse model of chronic HIV-related nephropathy associated with progressive fibrosis [42]. We have recently identified up-regulated FGF-2 protein and its mRNA in interstitial and tubular epithelial cells in human kidneys with tubulointerstitial scarring [17]. In that study, interstitial FGF-2 staining correlated with tubular and interstitial proliferative activity pointing to a pro-mitogenic effect in vivo.

This study demonstrates that FGF-2 may induce EMT in tubular epithelial cells in vitro. Similar to TGF-β1, FGF-2 reduced expression of cytokeratin and E-cadherin and induced expression of vimentin, FSP1 and SMA. Though TGF-β1 by itself had the most profound effects on cytokeratin and E-cadherin expression, its effects were substantially potentiated by FGF-2. E-cadherin represents the principal epithelial cell-cell adhesion molecule and is responsible for the integrity of epithelial layers [43]. The epithelial cell line Madin-Darby canine kidney (MDCK) disaggregates and converts into fibroblast-like cells after treatment with neutralizing antibodies to E-cadherin [44]. Loss of epithelial adhesion properties represents an early event in the process of EMT [31] that is sequentially followed by the loss of cytoskeletal epithelial and the acquisition of mesenchymal protein expression before completion [14]. The modest down-regulatory effects of all three cytokines were complemented by robust inhibitory effects on the E-cadherin promoter. We chose an upstream fragment (positions −214 to +92) since it displayed the highest epithelial specificity in kidney cells. This region is known to contain a GC-rich region that generates basic epithelial promoter activity and a palindromic sequence that potentiates that activity [44]. The effects of all three cytokines were more robust on the promoter than on the protein possibly due to long protein half-life [24]. However, even the promoter activity for E-cadherin was not completely abolished, indicating that the process of EMT cannot be induced to completion by one cytokine alone but requires an interplay of various growth factors. Furthermore, we chose to analyze far lower concentrations than the ones applied by Fan et al [30].

Conversely to epithelial cell markers, expressions of FSP1 and SMA were up-regulated by FGF-2. The up-regulatory effect on FSP1 expression was paralleled by stimulation of the FSP1 promoter. We studied an 1800 bp fragment with proven fibroblast specificity containing a fibroblast specific transcription site [11, 21]. The effects on FSP1 promoter activity were robust, but again a combination of cytokines was more efficient than each cytokine alone. We did not study the exact mechanisms of cytokine stimulation (or inhibition in the case of the E-cadherin promoter) since this was beyond the purpose of this study, but further studies using the stably transfected cell lines will permit us these analyses.

Interestingly, the three cytokines did not act synergistically on the expression of all markers. FGF-2, for example, induced de novo expression of OB-cadherin in NP-1 cells, unlike TGF-β1. OB-cadherin belongs to the family of atypical cadherins [45]. The structural features of this family resemble those of the classical cadherins with the exception that the classical cadherins lack the cell adhesion recognition sequence His-Ala-Val in the first extracellular domain [46]. The biological function of OB-cadherin has not been determined in its entirety, however, it may play a role in calcium-dependent cell adhesion of mesenchymal cells, for example in osteoblasts where it was first described. Osteoblasts may be derived from bone marrow fibroblasts and it is of particular interest in that regard that FGF-2 may induce such a differentiation step [47]. The description of OB-cadherin in tubular epithelial cells and its induction is novel and warrants further analysis.

Loss of integrity of the basement membrane is an important part of EMT. When MDCK cells are forced from the surface of the basement membrane they grow on, they develop mesenchymal characteristics [48]. Ng and coworkers localized tubular epithelial cells undergoing EMT as indicated by expression of SMA in the vicinity of tubular basement membrane disruption [6]. That the composition of the tubular basement membrane has important effects on the cellular phenotype was recently demonstrated by our group [49]. Thus, inducing metalloproteinases that may degrade basement membranes may play an important role in facilitating the process of EMT. FGF-2 increased secretion of MMP-2 and MMP-9 particularly in the distal tubular cell line. These two gelatinases participate in the degradation of the basal membrane, though extracellular activation is still required that was not induced in our study.

Basic fibroblast growth factor-2 had no effect on secretion of the ECM proteins collagen type I and fibronectin though intracellular fibronectin synthesis was stimulated. It is not clear why FGF-2 (and EGF) stimulate fibronectin synthesis but not secretion. In our study, TGF-β1 by itself had the most profound effect on secretion of fibronectin and collagen types I and IV. Interestingly, secretion of type I collagen could exclusively be stimulated in the distal tubular epithelial cell line.

That tubular epithelial cells are susceptible to the effects of FGF-2 was already demonstrated by Migdal and coworkers. Overexpression of FGFR-1 and addition of FGF-2 resulted in the acquisition of a fibroblastoid morphology and changes in the intracellular actin distribution [50]. Still, the potential of FGF-2 to induce EMT may seem surprising since studies by Perantoni et al and Sakurai and colleagues suggested that it promotes early tubulogenesis in embryonic development [51, 52]. However, it cannot promote epithelial conversion and may in fact inhibit tubulogenesis as was demonstrated by Dudley et al [53].

The in vitro changes observed in our study do not mean that these cells undergo complete and irreversible transdifferentiation. Regenerating tubular epithelial cells, for example, transiently express vimentin [54]. However, the chronic inflammatory processes and destruction of tubular basement membranes in chronic progressive renal disease may make a reversal to an epithelial cell type less likely. Moreover, as nicely demonstrated by Yang and Liu, the same changes described in our study in vitro can be observed in vivo including loss of epithelial cell adhesion markers, de novo SMA expression, disruption of the tubular basement membrane and cell migration [31].

In summary, our results demonstrate that FGF-2 promotes conversion of tubular epithelium to a cell with mesenchymal characteristics. Its effects are similar to EGF with the exception of cytokeratin and SMA expression where FGF-2 induces more profound changes. The effects on epithelial and mesenchymal marker proteins seem to be regulated on the promoter level. One important contribution of FGF-2 to EMT is the stimulation of microenvironmental proteases. Finally, the effects of FGF-2 are potentiated by additional incubation with TGF-β1.

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APPENDIX

Abbreviations used in this article are: ATP, adenosine triphosphate; DBA, *Dolichus biflorus* agglutinin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGTA, etyleneglycoltetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial mesenchymal transformation; FCS, fetal calf serum; FGF-2, basic fibroblast growth factor-2; FGFR, fibroblast growth factor receptor; FSP, fibroblast specific protein; Ig, immunoglobulin; kD, kilo Dalton; LTA, Lotus tetraglonolobus; MMP, matrix metalloproteinase; nAb, neutralizing antibody; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; SEM, standard error of mean; SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β.

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